Minimum Detectable Level of *Salmonellae* Using a Binomial-Based Bacterial Ice Nucleation Detection Assay (BIND®)

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A modified bacterial ice nucleation detection (BIND®) assay was used for rapid and sensitive detection of several *Salmonella* species. For the BIND assay, *Salmonella* cells are infected with bacteriophage genetically modified to contain DNA encoding an ice nucleation protein (INP). After infection, *de novo* protein synthesis occurs and INPs are incorporated into the outer membrane of the organism. After supercooling (–9.3°C), only buffer solutions containing transfected salmonellae freeze, causing a phase-sensitive dye to change color. This technique, and a probability-based protocol modification, provided quantitative detection with a minimum detectable level (MDL) of 2.0 ± 0.3 *S. enteritidis* cells/mL in buffer (about 3 h). The MDLs for *S. typhimurium* DT104 and *S. abaetetuba* were 4.2 ± 0.2 and 11.1 ± 0.4 cells/mL, respectively. Using salmonellae-specific immunomagnetic bead separation technology in conjunction with the modified BIND protocol, we achieved an MDL of about 4.5 *S. enteritidis* cells/mL with an apparent capture efficiency of 56%.

Most microbial plate counting techniques take advantage of the unique physiological characteristics and rapid reproductive turnover of microorganisms to provide simple, yet sensitive (400–2000 colony forming units [CFU]/mL) and selective, numerical assays. Cells are grown on selective media until organisms form a visually perceptible colony representing one CFU. Selectivity is gained by propagating such organisms on media which either allows growth of certain strains only or causes the colonies of interest to differ visually (e.g., Rainbow agar for *Salmonella*). These typical assays require several days. However, sensitive numerical results using simple and inexpensive equipment are often needed within 24 h. A number of rapid methods (1–3) have been developed to address some of these needs: antibody-direct epifluorescence microscopy (minimum detectable level [MDL] about 20 CFU/mL, sample throughput 1 h [4]); enzyme-linked immunomagnetic electrochemistry (about 10^3 CFU/mL, 80 min [5]); filtration-immuno-electrochemistry (about 10^4 CFU/mL, 25 min [6]); immuno-electrochemistry (about 10 CFU/mL, 10 min [7]); immuno-ligand assay/light addressable potentiometry (about 10^3 CFU/mL, 45 min [8]); immuno-magnetic-electro-chemiluminescence (about 10^3 CFU/mL, 1 h [9]); and multiplex polymerase chain reaction (about 10^2 CFU/mL, several h [10]). The weaknesses of most of these rapid methods are that their MDL is high (about 10^2–10^4 cells/mL), they demand relatively expensive instrumentation, and/or they require highly trained technical personnel to perform somewhat labor-intensive procedures. Additionally, most of these techniques do not discriminate between live or dead cells.

A recent study has implicated various *Salmonella* spp. as being responsible for numerous cases of food poisoning each year in the United States (11). The present study addresses concerns about the contamination of foods by *Salmonella* and approaches the goal of sensitive (viable cells), specific, yet simple detection methods. We have developed a probability-based bacterial ice nucleation detection (BIND®) protocol in which *S. enteritidis* was quantitated with an MDL of about 2 live bacteria cells/mL in 20 min after an initial 2.5 h incubation. Using this assay, the manufacturer (IDEXX, Inc., Westbrook, ME) has successfully detected >500 *Salmonella* isolates, 35 of which are recognized by the Centers for Disease Control and Prevention (CDC) as the predominant *Salmonella* spp. associated with food poisoning. However, the BIND assay, like the industry standard enzyme-linked immunosorbent assay (ELISA), was designed to determine only the presence or absence of pathogens post enrichment. In our quantitative approach, the number of *Salmonella*-positive wells, p, was determined from BIND and compared with the total aerobic plate count, δ, in order to determine if the bacteriophage-induced ice nucleation process is sensitive to 1 cell or CFU of *Salmonella* per aliquot of sample used

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(45 µL per well). We also demonstrate that the BIND protocol is easily modified for use with immunomagnetic bead (IMB) technology.

Experimental

Materials and Apparatus

The biological materials used in this research included *Salmonella enteritidis* (provided by K. Rajkowski, U.S. Department of Agriculture), *S. typhimurium* (H3380 California; serotype DT104, acquired from P. Hayes, CDC; clinical source), and *S. abaetetuba* (11:k:1,5; ATCC strain 35640; source: creek water from Zaiman, Argentina). Brain-heart infusion agar (BHIA) and broth (BHI) were obtained from Difco Laboratories (Detroit, MI). Rainbow agar (RA, selective for hydrogen sulfide-producing strains of *Salmonella*) was obtained from Biolog, Inc. (Hayward, CA). BIND assay kits were purchased from IDEXX, Inc. Other chemicals used were reagent grade.

All reactions requiring shaking were performed on a Vortex-Genie 2 (Scientific Industries, Bohemia, NY). The BIND Supercooler was obtained from IDEXX, Inc. Plating on both BHIA and RA was performed on an Autoplate 7000 (Spiral Biotech; 50 µL per 10 cm plate) apparatus. Only serially diluted samples estimated to contain 600–2000 CFU/mL were used for plate enumeration (i.e., 30–100 CFU per Petri dish from a starting concentration of ca 6×10^9/mL).

Most Probable Number (MPN) Estimation of Cell Density

Most MPN methods (12) involve: diluting the material to be tested, inoculating equal volumes of the dilutions into liquid media, and observing if growth occurs (turbidity) after incubation for 16–24 h. If numerous observations are to be made on the same dilution (as with binomial 96-well plate assays), the total probability, P (12, 13), that an organism will be contained in a certain volume is

$$P = \frac{n!}{p!q!}\left(e^{-v\Delta}\right)^q\left(1-e^{-v\Delta}\right)^p$$

In this relationship, v is the assay volume, p is the number of positive observations, q is the number of negative observations ($p + q = n$), and Δ is the number of organisms predicted to be in v (e.g., the MPN value). By setting the derivative of P with respect to Δ equal to 0, it is possible to find the value of Δ that corresponds to the maximum value of P

$$\frac{\partial P}{\partial \Delta} = \frac{vn!(e^{-v\Delta})^q(1-e^{-v\Delta})^p[q(e^{v\Delta}-1)-p]}{p!q!(e^{v\Delta}-1)} = 0$$

solving for Δ (there is only one meaningful solution), rearrangement, and simplification yields

$$\Delta = \frac{\log_e \frac{n}{n-p}}{v}$$

(cells/mL). For this MPN calculation to operate correctly, the solutions to be tested must be diluted so that p is always less than n. MPN tables (14) are based on more complicated expressions derived from similar probabilistic functions related to the presence or absence of organisms in a series of nested dilutions (e.g., 10^0, 10^{-1}, 10^{-2}) or assay volumes (12).

Minimum Detectable Level of Bacteria for Any Binomial-Based, n-Well Assay

After sufficient dilution, our sampling scenario allows only 2 possible outcomes: the sampled volume either does (p, positive) or does not (q, negative) contain the organism. Assuming that our sampling has been random, we have a binomial population (15). Suppose we define P_- as the probability that a subsample (v) from the total sample volume (V_{total}) will *not* contain the organism. For any assayed volume v (12)

$$P_- = (1-v_{org.}\delta)^{\frac{v}{v_{org.}}}$$

where v_{org} is volume of the test organism (about 2×10^{-13} mL) and δ is the cell density (cells/mL). Taking the natural logarithm of each side and using the appropriate series expansion of logarithms

$$\log_e P_- = -v\delta\left(1+\frac{v_{org}\delta}{2}+\frac{(v_{org}\delta)^2}{3}+\frac{(v_{org}\delta)^3}{4}+\cdots\right)$$

Because v_{org} is close to zero this reduces to

$$\log_e P_- = -v\delta$$

and

$$P_- = e^{-v\delta}$$

For p positive observations out of n total

$$p = n(1-P_-)$$
$$= n(1-e^{-v\delta})$$

In practice, we have found that the above equation requires a modification, as the agreement between the observed p and δ is not perfect. Therefore, we have inserted a non-fixed parameter, the correction factor µ, into the exponent of the above equation. This correction factor is mathematically equivalent to the slope of Δ (the most probable number, or binomial, estimation of cell concentration) when plotted as a function of δ (cell concentration from total aerobic plate count). Thus, µ is unity if 1 cell (or CFU) per unit volume v causes a positive response (e.g., ice nucleation, turbidity, etc.). Consequently, µ corrects for the observed number of positive responses not corresponding exactly to the theoretical relationship with regard to total aerobic plate count (δ); n and v are fixed parameters (for our work n = 96 and v = 45–50 µL)

$$p = n(1-e^{-v\mu\delta})$$

The estimation of μ is based on using nonlinear regression (e.g., fitting observed values of p, resulting from testing buffer solutions containing cells of density δ, as a function of δ) using a modified Gauss-Newton formalism (16) on a Microsoft EXCEL spreadsheet (17). We have performed these same calculations using traditional multiple dilution MPN computations and achieved identical values for μ (e.g., in this case $\mu = \Delta \div \delta$).

Enumeration of Salmonella Using 96-Well Binomial Plate Assays

The BIND Assay.—The standard BIND protocol, recommended by the manufacturer, involves culturing *Salmonella* isolates at 37°C for 16 h in buffered peptone water (BPW), serial dilution in BPW, distributing aliquots (0.5 mL) into either BIND sample tubes (12 × 75 mm polypropylene) which contain lyophilized bacteriophage, 5-(6)-carboxyfluorescein, HEPES/Tris, pH 7.5, and microbiological grade skim milk concentrate or BIND background tubes that contain all reagents except the bacteriophage. The tubes were agitated to dissolve the BIND pellets and incubated at room temperature (not to exceed 25°C) for 2.5 h. The incubated solutions were dispensed (50 µL each) into 1 row (8 wells per dilution) of an 8 × 2 well plate and supercooled at –9.3°C for 20 min; any phase-sensitive color change (yellowish-green to orange-red, indicating a positive response) upon freezing was logged as a positive (p) response.

Our modified protocol provided a thorough statistical sampling of bacterial dilutions. *Salmonella* cells, collected from a slant, were used to inoculate 25 mL BHI and incubated at 37°C for 16 h with shaking (160 rpm). The cells ($\bar{x} \pm s_{\bar{x}}$ [6 ± 1] × 10^9 CFU/mL) were serially diluted in filter-sterilized TRIS-buffered saline (TBS: 25mM TRIS, 150mM NaCl, and 10mM glucose; pH 7.6); one dilution was spiral-plated (50 µL each) onto both BHIA and RA Petri dishes (3–6 replicates each) and cultured overnight for enumeration (30–100 colonies each). Other serially diluted samples (ca 5 mL), estimated to contain between 5 and 80 CFU/mL, were placed in polycarbonate centrifuge tubes (2.5 × 10 cm) containing either pooled BIND sample or background reagents (10 pellets per tube) and vortex mixed. After 2.5 h reaction, 96 × 45 µL sample was evenly distributed among wells of a Falcon brand (Becton Dickinson Labware, Franklin Lakes, NJ) (round bottom, polystyrene) 96-well plate, sealed with clear adhesive film, and placed into the BIND supercooler set at –9.3°C. After 20 min, the number of positive responses,

$$p = p_{sample} - p_{background}$$

were counted; $p_{background}$ is the number of positive responses from the blank (TBS alone).

The Micro-MPN Turbidity Assay.—Another 96-well method, the micro-MPN turbidity assay, was used as a control binomial enumeration process with the BIND assay when immunomagnetic bead (IMB) technology was used. This control assay was added to compare relative capture efficiency of IMBs with the BIND assay. For every *Salmonella* concentration, 150 µL sterile BHI was pipetted into each reservoir of sterile 96-well plates; 50 µL sample (either TBS alone, TBS-diluted cells, immunomagnetically captured cells from TBS, or cells remaining in TBS after immunomagnetic separation; 0–50 CFU/mL) was then added (e.g., 96 × 50 µL per sample). All experimental manipulations were performed in a Type II Vertical Microbiological Hood. Inoculated plates were stored in a sterile container and incubated overnight at 37°C. After 16–24 h, the number of positive responses (p) was based on presence or absence of visual turbidity; there was complete agreement between visual turbidity and optical density determination on a Perkin-Elmer HTS7000+ plate reader.

Figure 1 shows a typical micro-MPN turbidity experiment (replicated 3 times). The uppermost inset figure displays the observed number of positive wells, p (out of n = 96), as a function of total aerobic plate count determined *S. enteritidis* cell density, δ (CFU/mL). The solid curve in Figure 1 (upper inset) represents the best fit and shows an excellent agreement with expected values for μ ($\bar{\mu} \pm s_{\bar{\mu}} = 1.01 \pm 0.004$; $\mu_{fit} \pm \varepsilon_{\mu} = 1.12 \pm 0.04$) ; the lowermost inset is the deviation in p across all replicates and δ values. Figure 1 also displays the calculated single dilution MPN values, Δ, as a function of δ, where the average deviation in predicted from observed was only ±0.13/mL and the slope (μ) was 0.996 ± 0.013. The results of another experiment (Table 1) compare the micro-MPN turbidity assay (Δ_{μ}) with the nested 1:10 multiple dilution (Δ_{nested} (14); n = 5, v = 1 mL from dilutions of 1, 0.1, 0.01, 0.001, 0.0001 in a total volume of 10 mL) MPN technique, showing that the former is about as accurate an estimator of cell density as the normal MPN protocol. In Table 1, the χ^2 statistic (5.8 and 2.1 for Δ_{nested} and Δ_{μ}; the lower the value of χ^2 the better the agreement, [15]) is provided as a measure of overall goodness of cell density prediction across the 4 concentration levels (3 degrees of freedom). The micro-MPN turbidity assay has several distinct advantages over the nested 1:10 dilution method inasmuch as it is much faster to perform, uses less media, results in less waste, and takes up less incubator space.

Immunomagnetic Bead (IMB) Capture.—From serial dilutions of *S. enteritidis* in TBS, 1 mL each was dispensed into five 1.5 mL centrifuge tubes. To each tube, 20 µL (1.34×10^7 beads) Dynal *Salmonella* IMBs were added and agitated 30 min at 25°C. The IMBs were magnetically isolated 5 min (the solution remaining after IMB isolation was saved for analysis), washed with cold TBS, and magnetically isolated again; the wash was then discarded. To each tube, 1 mL sterile TBS was added back to each tube and vortex mixed to resuspend the IMB-captured *Salmonella* cells. All samples were kept on ice between use. Thereafter, every 20 min, five 1 mL samples, representing each concentration of *S. enteritidis*, were combined in either a polycarbonate centrifuge tube (containing pooled BIND sample or background reagents) for the BIND assay or in a sterile reagent reservoir (for multichannel pipette dispensing) for the micro-MPN turbidity assay. All assays were also performed on the buffer alone after treatment with IMBs as additional control.

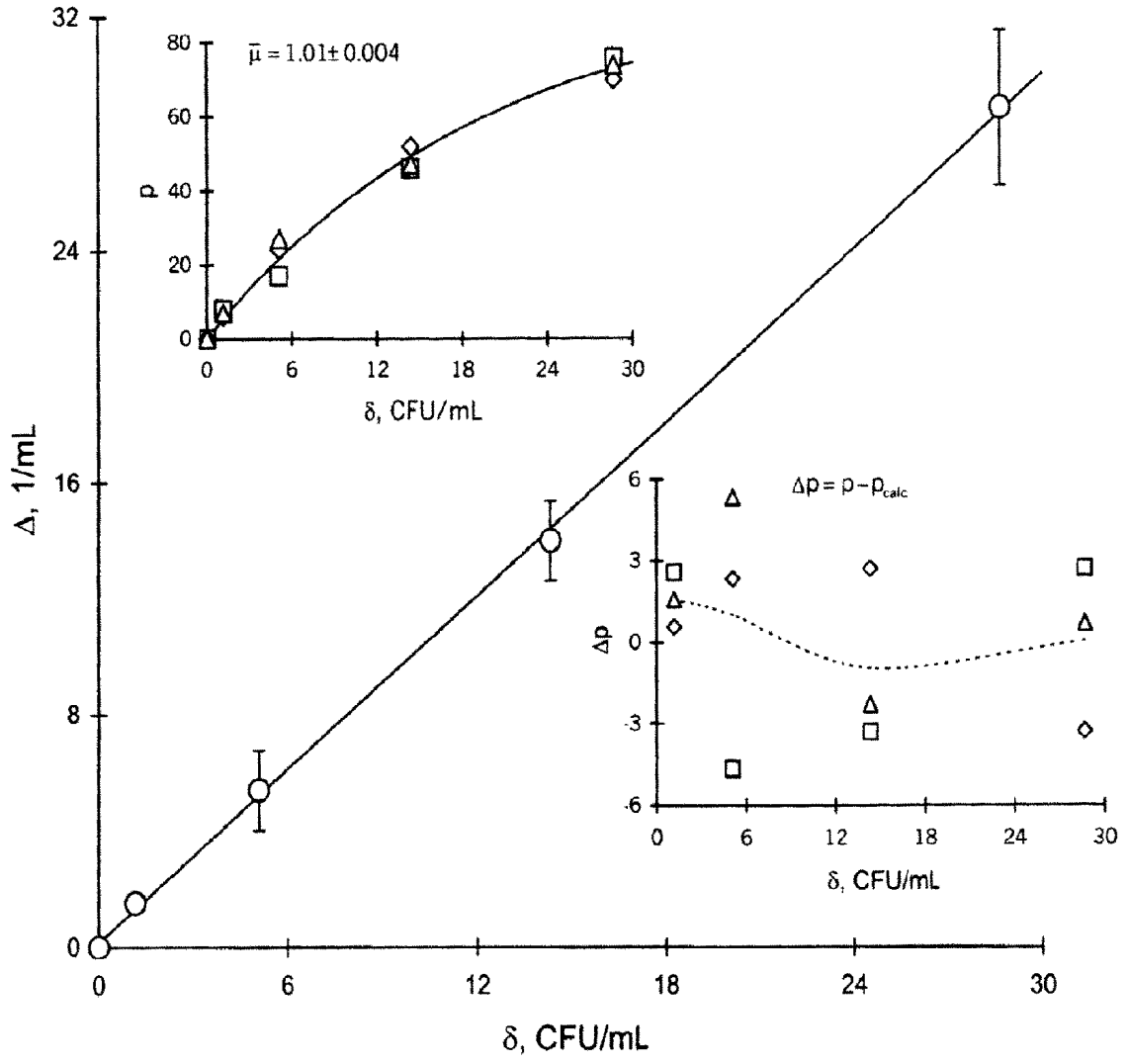

Figure 1. Correspondence between Δ ($\bar{\Delta} \pm S_{\Delta}^{-}$; from the micro-MPN turbidity assays) and bacterial density (δ, *Salmonella enteritidis*; CFU/mL) as determined by spiral plate culturing on BHIA. Upper inset: triplicate micro-MPN turbidity assay positive responses (p; n = 96) as a function of δ; the binomial fitting equation converged with μ = 1.12 ± 0.04. Lower inset: relative deviation in observed p from theory (e.g., μ = 1) for all replicates as a function of δ.

Table 1. Comparison of expected and observed estimations of cell density for the traditional multiple dilution MPN (Δ_{nested}) and single dilution micro-MPN (Δ_{μ}) methods with total aerobic plate count estimations of cell density (δ)

δ, CFU/mL	Δ_{nested}, cells/mL[a]	$\frac{(\Delta_{obs} - \Delta_{expected})^2}{\Delta_{expected}}$	cells/Δ_{μ}, mL[b]	$\frac{(\Delta_{obs} - \Delta_{expected})^2}{\Delta_{expected}}$
22.71	20.33	0.3025	17.02	1.3831
11.36	14.67	0.0317	14.28	0.6700
4.03	2.50	1.6364	3.90	0.0156
0.92	2.55	3.8281	0.63	0.0549
		χ^2: 5.7987		2.1236

[a] Δ_{nested} derived from MPN tables published in ref. 14 (multiple dilutions for each δ); for the $\Delta_{nested\text{-}expected}$ calculation, an array of calculated $p_{expected}$ from the 3-dilution (each 1:10) MPN table in reference 14.

[b] Δ_{μ} calculated from the single dilution MPN equation (= $\log_e$ {n/[n-p]}/v).

Results and Discussion

Application of the BIND assay in a format that enhanced statistical significance and sensitivity of results displayed nearly single organism detection of *S. enteritidis* (Figure 2; cells were cultured on separate days and serially diluted in TBS). The number of positive responses out of a possible n = 96 were plotted as a function of the total aerobic plate count (δ in units of CFU/mL). The modified BIND assay format was also applied (Figure 2) to the detection of *S. typhimurium* DT104 and *S. abaetetuba*. The exponential curve-fitting function (solid lines in Figure 2)

$$p_\delta = n(1-e^{-v\mu\delta})$$

was used because BIND data were mainly curvilinear for *S. enteritidis* beyond δ = 10 CFU/mL, had an origin at zero, and, most importantly, p_δ was theoretically implied by a probabilistic rationale (*see* **Experimental**). Note that p_δ worked equally well on nearly linear data, where the initial slope (= μnv), of the curved line was roughly equivalent to the slope (m) from linear regression analysis of the entire data set. For instance, using the *S. abaetetuba* BIND data (m = 0.3; μnv = 0.28), the solid curve (p_δ) was nearly congruent with the dashed line (μnv δ). A plot of the MPN-derived cell density, Δ (normalized with μ) as a function of δ, is displayed in Figure 3 for all *Salmonella* BIND data. These data were fairly linear (r^2 = 0.95) with a near-zero origin and slope near unity, and demonstrate that, knowing μ, the BIND protocol provides reasonably accurate estimates of δ. As a laboratory tool, knowing only μ, n, and v, one could estimate the *Salmonella* cell density. Practically (e.g., working with an unknown), to correct for the variable response of the BIND assay would be impossible without knowledge of the strain to be tested.

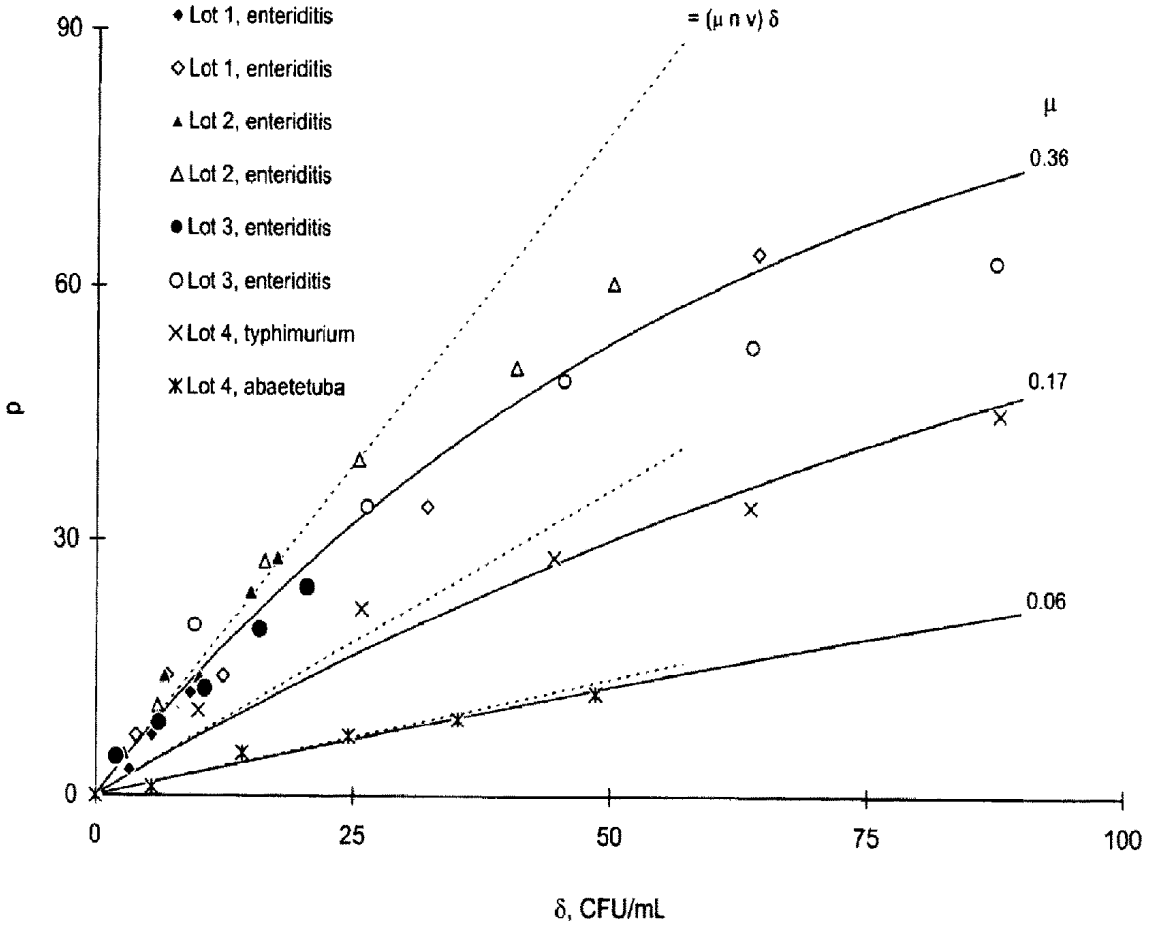

Figure 2. Detection of various *Salmonella* spp. using the modified BIND assay. Cells were cultured overnight in BHI broth, serially diluted in TBS, reacted with BIND assay (or background) reagent, and analyzed for the presence of ice nucleation activity. The plot displays the observed positive responses (p = p_{sample} - $p_{background}$) based upon BIND assay results versus organism concentration (δ, CFU/mL) as determined by spiral plate culturing on BHI agar. The dashed lines are equal to $\delta \left.\frac{\partial P_\delta}{\partial \delta}\right|_{\delta \to 0}$ and illustrate the effect of diminishing μ (provided adjacent to each curve) on the shape of the function. Different symbols refer to diverse *Salmonella* spp. as well as dissimilar lots of the BIND reagents.

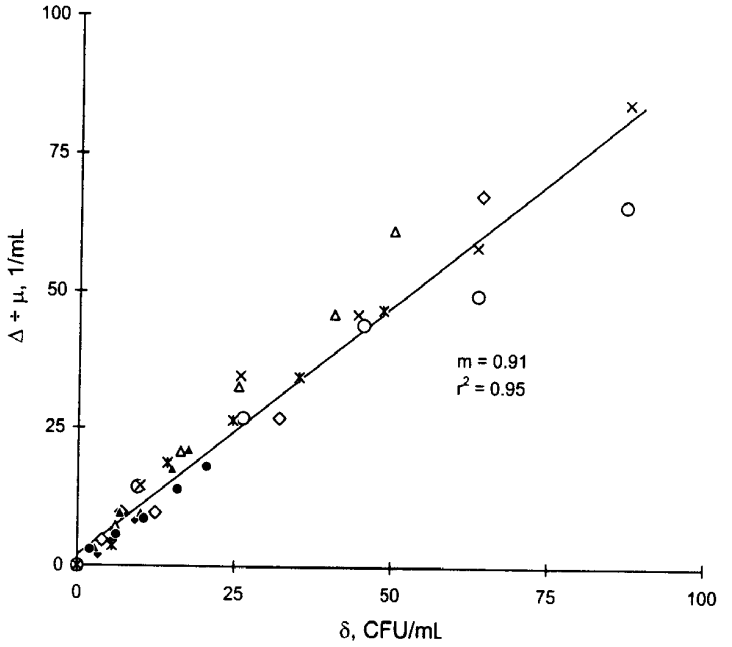

Figure 3. Plot of the MPN-derived values (Δ, cells/mL) of cell concentration versus total aerobic plate count (δ, CFU/mL) for the 3 *Salmonella* species discussed in this work: *S. enteritidis*, *S. typhimurium* DT104, and *S. abaetetuba*. *See* Figure 1 legend for symbol definitions. In Figure 3, all MPN calculations were normalized by dividing with μ.

Estimated minimum detectable levels (MDL or δ_{min}) for the various *Salmonella* spp. assayed with the modified BIND format are shown in Table 2, where

$$\delta_{min} = \frac{\log_e \frac{n}{n - p_{min}}}{v\mu}$$

and

$$p_{min} = 1 + \{\bar{p}_{background} + (s_{\bar{p}_{background}} t_{0.05})\}$$

=3 (rounding off to the nearest whole number)

In this calculation $s_{\bar{p}_{background}}$ is the standard deviation of the mean, $\bar{p}_{background}$ (0.5 ± 0.79 positives out of n = 96 observations, averaged over all experiments reported), v is the volume used per well (45 μL), and $t_{0.05}$ is the Student's 2-tailed value of t (15) at the 0.05 level. This value of p_{min} seems reasonable because 68% of all BIND background plates were negative ($p_{background}$ = 0) whereas 16% had only 1 or 2 positive responses out of 96 observations. Thus, the modified BIND assay MDLs varied only from 1.6 to 2.3 CFU/mL for this *S. enteritidis* isolate across 6 replicates and 3 lots of BIND reagents. However, the BIND assay was considerably less sensitive to *S. typhimurium* DT104 and *S. abaetetuba* (e.g., 4.2 and 11.1 cells/mL) than to *S. enteritidis*; similar results were also obtained with a different *S. typhimurium* isolate (MDL = 9 cells/mL; data not shown).

The data in Figure 2 and Tables 2 and 3 illustrate a problematic aspect of the BIND assay: inter- and intra-species variation in response (e.g., variation in μ). Because the BIND assay does not discriminate between one serotype (intra-genus

Table 2. Estimated minimum detectable (MDL ± σ_{MDL}) level of various *Salmonella* spp. in TBS using the modified BIND assay. Each replicate is based on 4–6 different dilutions (e.g., 4–6 different plates) of the same initial concentration of organism. Six different sets of *S. enteritidis* dilution cells were performed on separate days and serially diluted in TBS

	Minimum detectable level, CFU/mL						
Salmonella spp.	1	2	3	4	5	6	$\bar{x} \pm s_x$
S. enteritidis	2.28 ± 0.14	2.02 ± 0.13	1.64 ± 0.08	1.60 ± 0.04	2.20 ± 0.07	2.28 ± 0.18	2.00 ± 0.31
S. Typhimurium (DT104)	4.22 ± 0.22						
S. abaetetuba	11.10 ± 0.40						

Table 3. Variation in μ and MDL for various *Salmonella* spp. Each serotype or strain was grown overnight, diluted, and tested for minimum sensitivity (e.g., response significantly greater than blanks)

Salmonella spp.	μ	MDL[a]
S. agona	0.037	19
S. alachua	1	1
S. cubana	0.008	89
S. give	0.009	74
S. hadar	0.008	89
S. heidelberg	0.001	670
S. java	0.003	226
S. lille	0.011	64
S. mbandaka	0.093	7
S. montevideo	0.255	3
S. tennessee	0.008	82

[a] Calculated for n = 96.

differences in *Salmonella* spp. are based on serotype) or isolate of *Salmonella* over any other, it can only be as sensitive (when an unknown is investigated) as its worst case response which, in these experiments, was *S. heidelberg*. Table 3 provides additional information concerning the apparent intra-taxa disparity in μ, where MDLs varied from about 670 CFU/mL for *S. heidelberg* to about 1 CFU/mL for *S. alachua*. Clearly, some serotypes have near-maximal (μ about 1; implies that one transfected cell per 45 μL can induce ice nucleation) responses, whereas others show much less sensitivity than even *S. abaetetuba* (μ about 0.001). This wide variation is likely due to serotype-related deviations in either resistance to bacteriophage binding, expression of the transfected genes, and/or cell death prior to intercalation of sufficient INPs into the outer cell membrane. Although we have not fully investigated intra-serovar variation in μ, preliminary studies indicate this could be up to an order of magnitude.

Immunomagnetic Bead Capture

Another obstacle to the pragmatic use of the BIND assay is that certain nonpathogenic bacteria, which can contain natu-

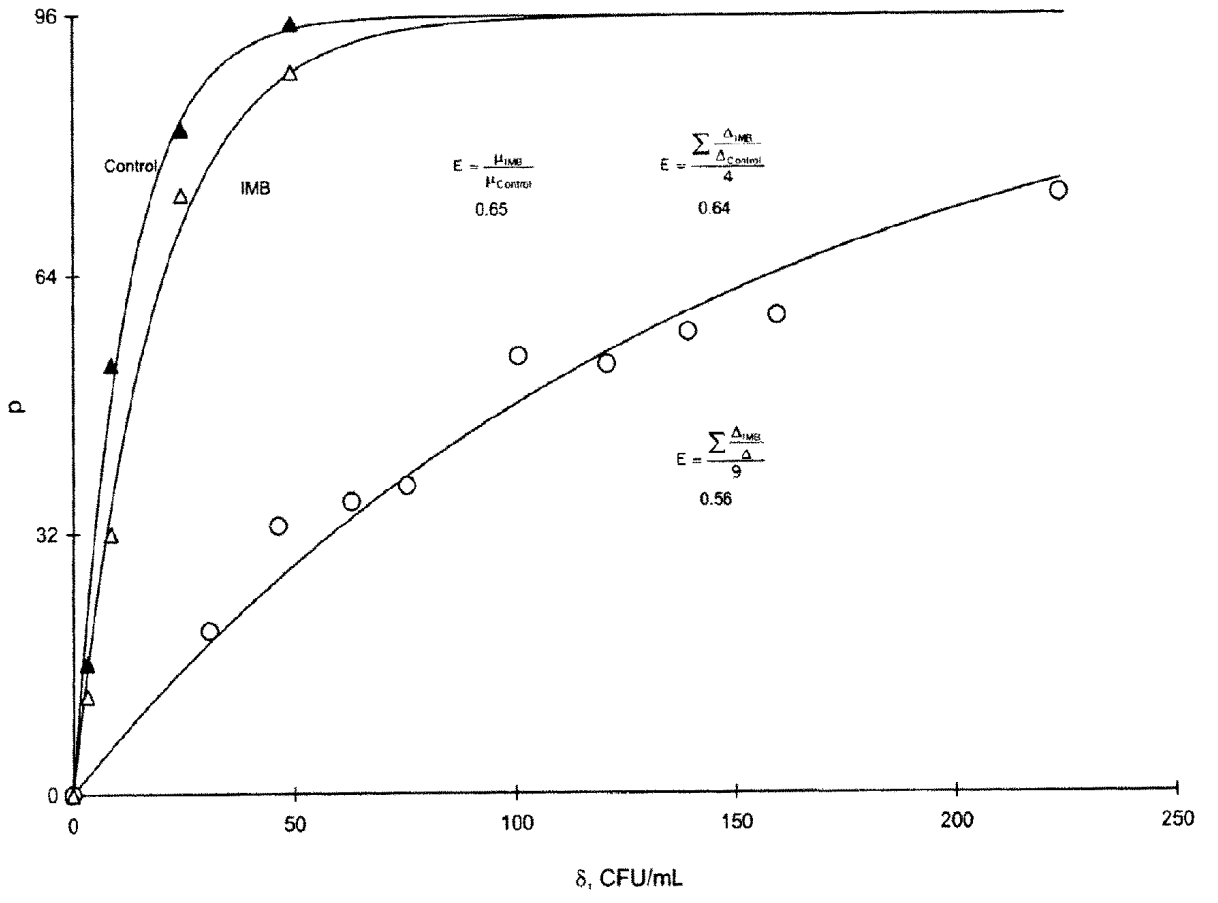

Figure 4. Relative response and capture efficiency of the micro-MPN turbidity assay (closed triangles = solution before IMB capture; open triangles = suspended IMBs) as compared with BIND enumeration (open circles) at various levels of δ (CFU/mL). The use of IMB capture diminished the MDL only by a factor of approximately 2.

rally occurring INPs in their outer walls, occur in real samples (e.g., carcass washes or other crude processing plant material), cause an increase in $p_{background}$, and thereby induce an unacceptable increase in the apparent MDL (data not shown; 18). To overcome this problem, the modified BIND assay must be used in conjunction with some selective separation procedure such as the well-known IMB separation method (19). To determine the effect of IMB separation on the MDL for *S. enteritidis*, we performed a series of experiments using both the BIND and micro-MPN turbidity assays. Figure 4 displays the results of these experiments in which we observed that the use of IMB capture decreased the sensitivity and increased the MDL from 2 to approximately 4.5 ± 0.18 CFU/mL. This decrease in sensitivity is due to inefficiency of the capture process under these conditions (e.g., 1.34×10^7 IMB/mL with 30 min mixing time). The ratio of BIND Δ values from the IMB experiments to the sum of both IMB and supernatant after IMB treatment (Δ_{IMB}/Δ, averaged over all concentrations), which is a measure of the overall capture efficiency, was 0.56 ± 0.1. This measure of efficiency (E) was not significantly different from E obtained from the binomial control micro-MPN turbidity assays (0.64 ± 0.12; Figure 4). Using nearly 3-fold more IMBs (*E. coli*, 10^6 CFU/mL) Perez and co-workers measured an apparent capture efficiency (20) of about 80%.

Conclusions

We have shown that binomial 96-well assays have the potential for very sensitive bacterial enumeration. The major weakness of the BIND assay relates to its variable response to different *Salmonella* isolates as well as high background responses with real samples because of the presence of other ice nucleating agents. These problems can be solved with the selective isolation of specific serotypes of *Salmonella* using technologies such as IMBs coated with monoclonal antibodies in conjunction with blocking reagents (5). The micro-MPN turbidity assay is also very sensitive and accurate but probably only useful for research purposes (pure strains; MDL = 0.2–0.4 CFU/mL) because it is nonselective.

The weaknesses of most other (5–10) enumeration methods is that either their MDL is high (about 10^2–10^4 cells/mL), they demand relatively expensive instrumentation, and/or they require highly trained technical personnel to perform the somewhat laborious and complicated procedures. Additionally, none of these techniques (4–10) discriminate between live or dead cells. The modified BIND assay, on the other hand, is relatively rapid with a sample throughput of approximately 16 per day per 8 × 12 well Supercooler. Although the cost per assay using our more extensive observational protocol (n = 96) is 12-fold greater than the standard protocol, the equipment required is inexpensive and simple to operate. Thus, the total cost per sample is relatively low when the savings in training and equipment are considered. By modifying our method for n = 24, one should be able to substantially reduce the cost and still achieve a reasonable MDL (about 16 CFU/mL for our *S. enteritidis* isolate with IMB capture).

Symbols and Abbreviations

BIND = bacterial ice nucleation detection
CFU = colony forming unit;
δ = cell concentration in TBS (units of CFU/mL or cells/mL) from total aerobic plate counts
Δ = cell concentration (units of cells/mL) from binomial assays as determined from an MPN table (i.e., Table 1, Δ_{nested}) or as

$$\Delta = \frac{\log_e \frac{n}{n-p}}{v} \text{ (i.e., Table 1, } \Delta_\mu)$$

ε_μ = asymptotic standard error (16, 17) for parameter μ determined from nonlinear regression analysis, using the modified Gauss-Newton method
μ = coefficient of agreement between cell enumeration via binomial assays and total aerobic plate count; perfect agreement yields a μ of 1 (see definition of p, below)
MDL = minimum detectable level
MPN = most probable number
n = number of observations per dilution in BIND or micro-MPN bioassays
p = number of positive responses in BIND or micro-MPN bioassays; in theory

$$p = n(1 - e^{-v\mu\delta})$$

q = number of negative responses (i.e., q = n – p) in BIND or micro-MPN bioassays
IMB = immunomagnetic beads
INP = ice nucleating protein
TBS = TRIS-buffered saline
v = volume of sample used per well or tube in BIND or micro-MPN bioassays

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References

(1) Fung, D.Y.C. (1995) *Food Technol.* **49**, 64–67
(2) Feng, P. (1992) *J. Food Prot.* **55**, 927–934
(3) Hartman, P.A., Swaminathan, B., Curiale, M.S., Firstenberg-Eden, R., Sharpe, A.N., Cox, N.A., Fung, D.Y.C., & Goldschmidt, M.C. (1992) in *Compendium of Methods for the Microbiological Examination of Foods*, 3rd Ed., C. Vanderzant & D.F. Splittstoesser (Eds), American Public Health Association, Washington, DC, pp 665–746

(4) Tortorello, M.L., & Stewart, D.S. (1994) *Appl. Environ. Microbiol.* **60**, 3553–3559

(5) Gehring, A.G., Crawford, C.G., Mazenko, R.S., Van Houten, L.J., & Brewster, J.D. (1996) *J. Immunol. Methods* **195**, 15–25

(6) Brewster, J.D., & Mazenko, R.S. (1998) *J. Immunol. Methods* **211**, 1–8

(7) Rishpon, J., Gezundhajt, Y., Soussan, L., Rosen-Margalit, I. & Hadas, E. (1992) *ACS Symp. Series* **511**, 59–72

(8) Gehring, A.G., Patterson, D.L., & Tu, S. (1998) *Anal. Biochem.* **258**, 293–298

(9) Yu, H., & Bruno, J.G. (1996) *Appl. Environ. Microbiol.* **62**, 587–592

(10) Deng, M.Y., & Fratamico, P.M. (1996) *J. Food Prot.* **59**, 570–576

(11) USDA, Food Safety and Inspection Service (1995) *Fed. Regist.* **60**, 6774–6903

(12) Halvorson, H.O., & Ziegler, N.R. (1933) *J. Bacteriol.* **25**, 101–121

(13) McCrady, M.H. (1915) *J. Infect. Dis.* **17**, 183–212

(14) de Man, J.C. (1975) *Europ. J. Appl. Microbiol.* **1**, 67–78

(15) Steel, R.G.D., & Torrie, J.H. (1960) in *Principles and Procedures of Statistics,* McGraw-Hill Book Co., Inc., New York, NY, pp 352–365

(16) Draper, N., & Smith, H. (1981) in *Applied Regression Analysis*, 2nd Ed., John Wiley & Sons, New York, NY, pp 462–465

(17) Irwin, P.L., Damert, W.C., & Doner, L.W. (1994) *Concepts Magn. Reson.* **6**, 57–67

(18) Peplow, M.O., Correa-Prisant, M., Stebbins, M.E., Jones, F., & Davies, P. (1999) *Appl. Environ. Microbiol.* **65**, 1055–1060

(19) Lea, T., Vartdal, F., Davies, C., & Ugelstad, J. (1985) *Scand. J. Immunol.* **22**, 207–216

(20) Peréz, F.G., Mascini, M., Tothill, I.E., & Turner, A.P.F. (1998) *Anal. Chem.* **70**, 2380–2386